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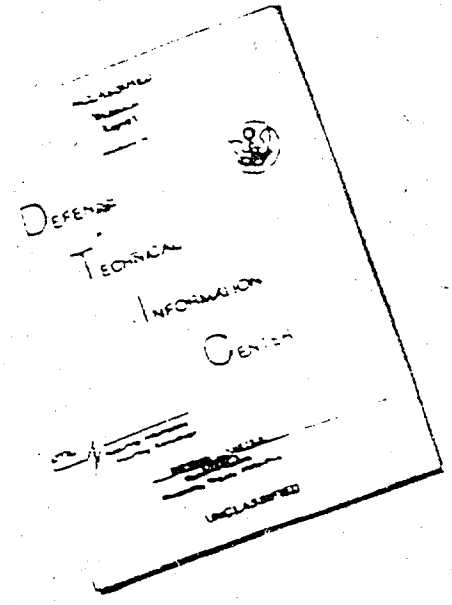

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TECHNICAL MANUSCRIPT 104

PATHOGENESIS OF EXPERIMENTAL RESPIRATORY TULAREMIA IN MONKEYS

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PATHOGENESIS OF EXPERIMENTAL TULAREMIA IN MONKEYS: EFFECT OF PARTICLE SIZE

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ABSTRACT

The purpose of the experiments described in this report was to determine the primary sites of infection and subsequent histogenesis of lesions in lungs of rhesus monkeys exposed to the SCHU S4 strain of Pasteurella tularensis in aerosols consisting of particles either one or eight microns in diameter. The monkeys were sacrificed at 1/3, 12, 24, 72, and 96 hours after exposure and tissues were examined by conventional pathological techniques and fluorescent antibody methods. Under the conditions of these experiments the respiratory bronchiole was the primary site of infection in the lung of rhesus monkeys. Intracellular P. tularensis was demonstrated in respiratory bronchioles by fluorescent antibody staining of tissues obtained 20 minutes after exposure to aerosols of one-micron particles.

I. INTRODUCTION

Although a considerable body of information has been published on pulmonary disease in man and animals, relatively few facts are available concerning the pathogenesis or histogenesis of the primary stages of infectious pulmonary diseases. Mitchell[1] has reviewed the literature on the deposition and fate of particles introduced into the respiratory system. The majority of information, however, deals with inert particles rather than particles containing viable microorganisms.

Druett and coworkers studied the relationship between particle size and infectivity of Pasteurella pestis[2] and Brucella suis[3] for guinea pigs and infectivity of anthrax spores[4] for guinea pigs and monkeys. For the size range of one to 12 microns, infectivity decreased as particle size increased. Additional information on the influence of particle size on infectivity is contained in the reviews by Fothergill[5] and Goodlow and Leonard.[6]

The purpose of the experiments reported here was to determine the primary sites of infection and subsequent histogenesis of lesions in lungs of rhesus monkeys exposed to aerosols of Pasteurella tularensis.* Refined methods[7] for disseminating, controlling, and assaying aerosols of viable, pathogenic microorganisms have made possible a correlation of the histogenesis of respiratory infection with various physical characteristics of the aerosol. This report also deals with the influence of particle size on the histogenesis of the primary lesions induced in Macaca mulatta by aerosols containing P. tularensis.

* Animals were maintained in compliance with the "Principles of Laboratory Animal Care" as promulgated by the National Society for Medical Research.

II. MATERIALS AND METHODS

A. CULTURE

The SCHU S4 strain of P. tularensis was grown in modified casein partial hydrolyzate (MCPH). Sterile MCPH, diluted with an equal volume of sterile distilled water, was added to the culture to make the desired bacterial concentration in the suspension used for generating the aerosol.

B. AEROSOLS

The infectious aerosols were disseminated into a modified Henderson apparatus by either a Collison spray head[8] or the vibrating reed.[9] Relative humidity of the aerosols ranged from 50 to 60 per cent and the temperature was 27°C. The diameter of 95 per cent of the particles generated by the Collison spray head was one micron or less and that of all particles generated by the vibrating reed was eight microns (variation ±0.1 micron).

To determine the concentration of viable air-borne organisms, a measured volume of the aerosol was drawn through midget Shipe impingers,[10] and the fluid within, gelatin-saline, was assayed by conventional bacteriological plating procedures for viable content. The dose for each monkey was then calculated by multiplying the bacterial concentration per liter of aerosol, the exposure period in minutes, and the respiratory volume. Respiratory volumes were determined on the basis of body weight and Guyton's formula.[11]

C. ANIMALS

Twenty-eight rhesus monkeys, Macaca mulatta, were used. They were caged in pairs and given Purina monkey chow and water ad libitum. The average body weight of this group of animals was 3.5 kilograms.

For exposure to the aerosol, each monkey was placed in a plastic holding chair and fitted with a helmet attached to the Henderson apparatus. The aerosol was drawn into the helmet by a regulated vacuum. Use of the holding chair permitted exposure without use of anesthesia. Sixteen monkeys were exposed to aerosol particles one micron in diameter for five minutes; the average dose was three million cells of P. tularensis. Twelve monkeys were exposed to aerosol particles eight microns in diameter for five minutes. The average dose was 1600 cells.

D. NECROPSIES

At selected times after exposure, animals were euthanasized by intravenous injection of Nembutal (Abbott)* and necropsied immediately. The schedule and the numbers of animals sacrificed at each time are shown in Table I.

TABLE I. NUMBER OF ANIMALS SACRIFICED AFTER EXPOSURE TO THE AEROSOLS

Diameter Particles in Aerosol, microns	Number of Animals Sacrificed Time after Exposure, hours					
	1/3	12	24	48	72	96
1	2	2	4	4	4	-
8	-	-	2	4	4	2

Samples of lung for quantitative bacteriological culture and frozen tissue for fluorescent antibody staining were obtained from four pairs of the animals exposed to the one-micron particles and sacrificed at 1/3, 24, 48, and 72 hours. Samples of lung were triturated in glass grinders, serially diluted in gelatin-saline, and 0.2-milliliter samples were spread on glucose-cysteine-blood-agar plates. Counts were made after incubation for 72 and 96 hours and total concentrations calculated. A sample of spleen, liver, tracheobronchial lymph nodes, and each lobe of the lungs was frozen at -70°C and stored at -20°C for fluorescent antibody studies.

The tissues and organs obtained from the remaining animals were fixed in ten per cent neutral formalin. Necropsies were done with sampling of the internal organs, including upper respiratory tract.

* Nembutal (Sodium) Veterinary, Abbott Laboratories, North Chicago, Ill.

The tissues fixed in formalin were processed through paraffin, cut at four micron, and stained with hematoxylin and eosin and a modified Giemsa method. Thirty to fifty sections of lung were examined from each animal. Coronal sections of the decalcified nasal cavities and nasopharynx were prepared.

E. FLUORESCENT ANTIBODY STAINING

Immune monkey serum with an agglutinin titer of 1:1280 for P. tularensis was fractioned with ammonium sulfate and conjugated with fluorescein isothiocyanate.[12] The conjugate was adsorbed twice with mouse liver powder (100 micrograms per milliliter) and twice with rabbit bone marrow. A portion of the conjugate was further adsorbed with washed, formalin-killed, P. tularensis to remove homologous antibody. This reagent, used for control, did not stain P. tularensis.

Sections of frozen tissues were placed in acetone for 30 minutes, dried in air, and stained 30 minutes with a 1:30 dilution of conjugate. A companion section was treated similarly but stained with the conjugate adsorbed with tularensis cells. When tissues fixed in formalin were used, the paraffin sections were hydrated in the usual manner, washed in buffered saline, and stained. All slides were examined with a Zeiss fluorescence microscope equipped with a 200-watt Osram lamp, Schott UG-2 and UG-5 transmitting filters, and a Schott GG-4 barrier filter.

III. RESULTS

A. CULTURAL RECOVERY OF P. TULARENSIS

At 20 minutes after exposure to the aerosol of one-micron particles, the concentration of bacteria in the lung tissue was 21,000 per gram. The total bacterial content, calculated by multiplying the concentration per gram by the weight of the lungs, was 378,000 cells. This represented a pneumonic retention of 12.6 per cent based on an inhaled dose of three million cells. The numbers of bacteria recovered from the lung at each time after exposure are presented in Table II.

TABLE II. NUMBERS OF P. TULARENSIS IN LUNGS OF MONKEYS EXPOSED TO AEROSOL OF ONE-MICRON PARTICLES

Time After Exposure, hours	Concentration	
	Per Gram	Total
1/3	2.1×10^4	3.8×10^5
24	2.9×10^8	3.8×10^7
48	4.0×10^8	9.5×10^8
72	1.9×10^9	7.0×10^{10}

B. GROSS OBSERVATIONS

Gross lesions were apparent at 72 hours in the lungs of animals exposed to the aerosol of one-micron particles. Numerous reddish, firm, focal lesions, 0.2 to 0.5 centimeter in diameter, were present in all lobes. The tracheobronchial lymph nodes were distinctly enlarged at 72 hours and focal areas of necrosis were seen on the cut surface.

The lungs of the animals exposed to eight-micron particles and sacrificed at 72 hours contained some areas of hyperemia. A few focal necrotic lesions, similar to those seen in the one-micron group, were present at 96 hours. The changes in the tracheobronchial lymph nodes were similar to those in the animals exposed to one-micron particles, but were not seen until 24 hours later.

Gross changes in the liver and spleen were apparent at 72 hours in the one-micron group and at 96 hours in the eight-micron group. The liver was a mottled yellowish tan and the borders were slightly rounded. The spleen appeared enlarged, and the cut surface was dry. A few minute, yellow, focal lesions were seen on the cut surfaces of these organs.

C. MICROSCOPICAL OBSERVATIONS*

The histological findings were similar in each exposure group, although the appearance of the lesions was delayed in the animals exposed to the aerosol of eight-micron particles.

The earliest reaction to infection as observed by light microscopy was found at 24 and 48 hours in the one- and eight-micron groups, respectively. However, by fluorescent antibody staining, P. tularensis was found in the lungs of animals killed 20 minutes after exposure to the aerosol of one-micron particles. When anatomical features could be identified in the frozen sections, the organisms were found in macrophages in the lumina of respiratory bronchioles (Figure 1). Organisms were detected in slightly less than ten per cent of the 160 slides examined from this group of animals.

At 24 hours, a focal bronchiolitis was apparent in the animals exposed to one-micron particles. The bronchiolitis was localized in the respiratory bronchiole, near its junction with the terminal bronchiole. The inflammatory reaction, predominantly neutrophiles and macrophages, involved the peribronchiolar tissues with exudation into the bronchiolar lumen (Figure 2). Pasteurella tularensis was found in the cytoplasm of macrophages in these lesions (Figure 3).

By 48 hours, a respiratory bronchiolitis, identical to that described above, was found in the animals exposed to the eight-micron particles. The incidence of lesions was much lower in this group of animals.

At 48 hours in the one-micron group and at 72 hours in the eight-micron group, the bronchiolitis was more pronounced. There was evidence of necrosis in the intraluminal exudate. The inflammatory reaction in the peribronchiolar tissues and within septa of the rudimentary alveoli in the respiratory bronchioles appeared to extend and involve the alveoli spaces (Figures 4 and 5). Pasteurella tularensis was readily identified in these lesions. In many areas, a peribronchiolar and perivascular lymphangitis was seen. Neutrophiles and P. tularensis cells were observed in the distended lymphatics (Figure 6).

An extensive confluent bronchopneumonia developed by 72 hours in the one-micron group and in 96 hours in the eight-micron group. Focal lesions developing in the peribronchial lymphatics and lymphoid tissue frequently appeared to invade the walls and break into the lumina of the bronchi.

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* All references to P. tularensis in this section are based on observations under the fluorescence microscope.

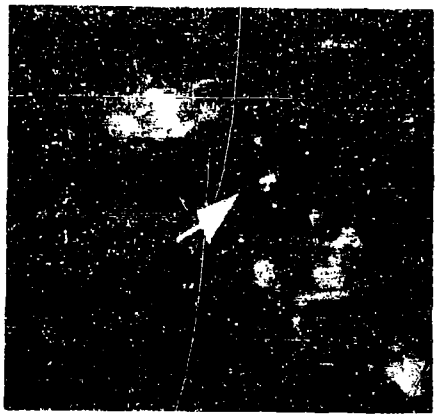

Figure 1. Section Through Respiratory Bronchiole of Monkey Killed 20 Minutes After Exposure to Aerosol of One-Micron Particles. The arrow points to a macrophage containing P. tularensis. 190X.

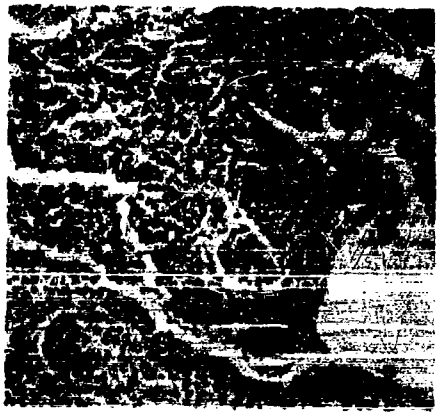

Figure 2. Section Through Respiratory Bronchiole of Monkey Killed 24 Hours After Exposure to Aerosol of One-Micron Particles. Note intraluminal exudate. 130X.

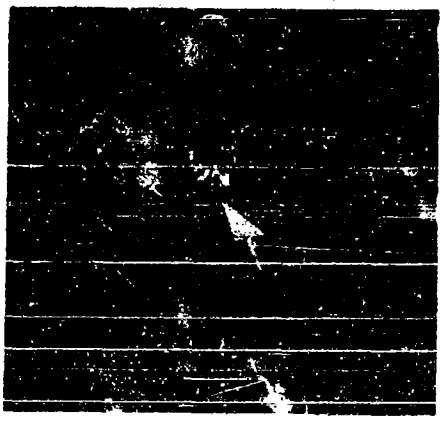

Figure 3. Twenty-Four Hours After Exposure to One-Micron Particles, Showing P. tularensis in Submucosa of Respiratory Bronchiole. 180X.

Figures 1 and 3 are frozen sections of lungs stained with fluorescent antibody. Figure 2 is section of lung embedded in paraffin and stained by a modified Giemsa method.

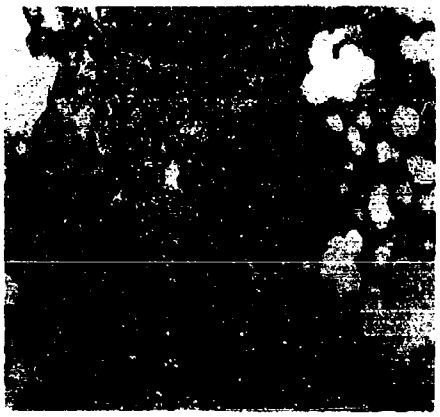

Figure 4. At 72 Hours After Exposure to One-Micron Particles, the Lesion has Expanded to Include Parenchyma Adjacent to Respiratory Bronchiole. 42X.

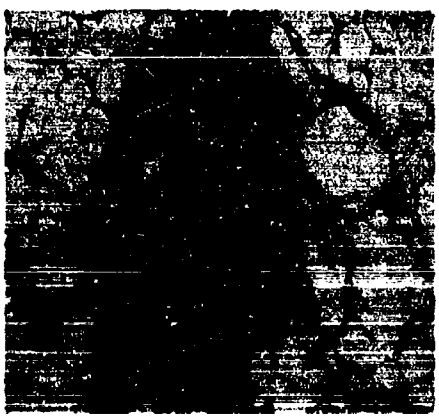

Figure 5. Section of Lung From Monkey Killed 72 Hours After Exposure to Eight-Micron Particles. 42X.

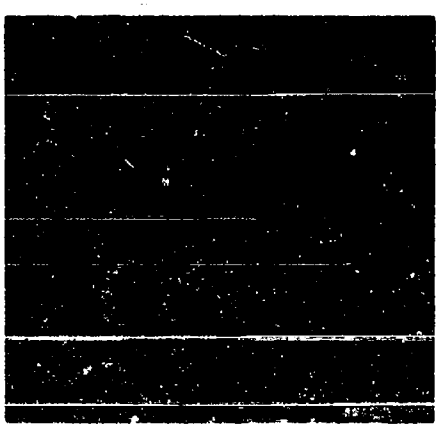

Figure 6. Perivascular Lesion with Cells of P. tularensis. Bacteria are seen in perivascular lymphatics. 100X.

Figures 4 and 5 are sections of lungs embedded in paraffin and stained by a modified Giemsa method. Figure 6 is frozen section of lung stained with fluorescent antibody.

Microscopical changes in the tracheobronchial lymph nodes were seen at 48 hours in the one-micron group and at 72 hours in the eight-micron group. Cortical foci of necrosis surrounded by neutrophiles and histiocytes were present. Cells of P. tularensis were found in these lesions. By 72 and 96 hours in the two groups, the normal architecture of the lymph nodes was replaced by confluent necrosis.

No tularemic lesions were found in the coronal sections of nasal and nasopharyngeal cavities. The liver and spleen had small focal lesions of necrosis with neutrophiles and macrophages by 24 hours in the one-micron group and 72 hours in the eight-micron group. Pasteurella tularensis was found in these sites of reaction.

IV. DISCUSSION

Under the conditions of these experiments, it is apparent that the respiratory bronchiole is the primary site of infection in the lung of rhesus monkeys exposed to aerosols of P. tularensis. In this study, the respiratory bronchiole was considered to be that portion of the bronchiolar system with: (a) small bundles of smooth muscle in the wall outlining the openings of small alveoli, (b) a low cuboidal or squamous, nonciliated, non-mucus-producing epithelium overlying the smooth muscle bundles, (c) an associated artery, (d) anthrocotic pigment in the immediate peribronchiolar tissue, and (e) evidence of direct connection with alveolar ducts.

Although the present work dealt only with the virulent SCHU S4 strain of P. tularensis, other experiments with monkeys have indicated that the respiratory bronchiole is the initial site of infection with the live vaccine strain of P. tularensis[13] and with psittacosis virus.[14] The susceptibility of the respiratory bronchiole to injury by nitrogen dioxide was shown by Kleinerman.[15] Schiller[16] is of the opinion that dust particles invade the walls of respiratory bronchioles.

Our experiments have not revealed the immediate site of deposition of infectious aerosol particles. The demonstration of intracellular P. tularensis by fluorescent antibody staining of tissue obtained 20 minutes after exposure may indicate that phagocytosis occurred either in the respiratory bronchiole or distally, followed by migration of the phagocyte up to the respiratory brochiole. Whichever is the case, the initial lesion occurs in the wall of the respiratory bronchiole.

It is apparent that particle size of the aerosols in our experiments did not influence the site of respiratory bronchiolitis. Within the diameters of one to eight microns, infectious particles containing P. tularensis induce respiratory bronchiolitis in rhesus monkeys. These data do not preclude the possibility that the larger particles break down within the airstream of the respiratory tract. Determination of the condition, size, or degree of intrapulmonary breakup or dispersion of the larger particles was not within the scope of this study.

Lesions of tularemic bronchiolitis were first detected in the monkeys exposed to the eight-micron particles at 48 hours in contrast to 24 hours for the monkeys exposed to one-micron particles. This difference may be attributed to the doses used. The highest practicable dose with the equipment available was employed to facilitate detection of organisms and lesions. Although 21 thousand bacteria per gram of lung were recovered approximately 20 minutes after exposure to one-micron particles, less than ten per cent of the tissue sections, four microns thick, contained P. tularensis. The dose contained in eight-micron particles was much lower; therefore, fewer organisms reached the respiratory bronchioles, resulting in fewer lesions. Thus, the apparent 24-hour delay in appearance of lesions in the animals exposed to eight-micron particles may have been due to the fact that the comparatively small number of lesions produced escaped earlier detection by the sampling techniques used. The lesions, however, when first detected were in respiratory bronchioles.

This is in contrast to the findings of Druett and coworkers,[8] who exposed guinea pigs to P. pestis in aerosol particles 12 microns in diameter or clouds of single organisms. The latter aerosols consisted of small particles that produced bronchopneumonia and a fatal septicemia. Animals that inhaled the large particles became septicemic and died in a shorter time, but did not have a pneumonia.

V. SUMMARY

Rhesus monkeys were exposed to the SCHU S4 strain of P. tularensis in aerosols consisting of particles either one or eight microns in diameter. Intracellular P. tularensis was demonstrated in respiratory bronchioles by fluorescent antibody staining of tissues obtained 20 minutes after exposure to aerosols of one-micron particles. The initial lesion, seen at 24 hours in the one-micron group and at 48 hours in the eight-micron group, was a respiratory bronchiolitis. This bronchiolitis appeared to extend into adjacent pulmonary tissue and involve the alveolar spaces. P. tularensis was readily identified in the bronchiolar and bronchopneumonic lesions.

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